INCREASED RNase H (HYBRIDASE) ACTIVITY IN THE INTEGUMENT OF BLOWFLY LARVAE DURING DEVELOPMENT AND UNDER THE INFLUENCE OF β-ECDYSONE

D. DOENECKE, V.J. MARMARAS and C.E. SEKERIS

Institut für Physiologische Chemie der Universität Marburg, 355 Marburg/Lahn, Lahnberge, W. Germany

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1. Introduction

Ribonuclease H or hybridase, an enzyme first described by Stein and Hausen [1] degrades specifically the RNA moiety of DNA/RNA hybrids. Its biological significance is still obscure. Due to the fact that one of the DNA-dependent RNA-polymerases of eukaryotes, polymerase B, transcribes *in vitro* preferentially single stranded DNA [2] forming RNase-resistant DNA/RNA hybrids which may also be formed *in vivo*, we have considered the possibility of the involvement of ribonuclease H in the control of transcription. One approach towards this goal was the assessment of hybridase activity in tissues which show dramatic changes in RNA synthesizing capacity, either due to developmental changes or to hormonal stimulation. In this respect the integument of blowfly larvae is ideally suited [3]. RNA synthesis of the integument is at a very low level in 6–7 day old larvae and increases dramatically in white prepupae. This increased rate of RNA synthesis is due to the action of the steroid hormone β-ecdysone, which is secreted at that time [4]. We therefore measured hybridase activity in the integument of 6–7 day old larvae in comparison to that of white prepupae as well as that of ligated animals injected with β-ecdysone. The results of these experiments are presented below.

2. Materials and methods

Calliphora erythrocephala larvae reared on bovine meat at 23° and 50% relative humidity, conditions under which the larval life lasts approx. 8 days, were used.

All reagents were analytical grade from Merck (Darmstadt, Germany), Sephadex was from Pharmacia (Uppsala, Sweden), DEAE-cellulose from Whatman (London, U.K.).

2.1. *Preparation of RNase H from the larval integument*

The enzyme is in principle prepared by the same procedure as the different forms of DNA-dependent RNA-polymerases from the larval tissue [5]. In the developmental experiments 6–7 day old larvae and white prepupae were compared. In the other set of experiments 6–7 day old larvae were ligated between the head and the abdomen. After pupation of the head 0.01 μg of β-ecdysone was injected into the hind part of the ligated animal, control animals received an injection of buffer. After 2 hr heads of control and injected animals were removed and the bodies treated as described below.

In parallel preparations 120 larvae of the different groups were washed in medium A (0.067 M Tris, 1 M $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 0.25 mM EDTA-Na_2, 1 mM mercaptoethanol, 10 mM NH_4Cl and 20% glycerol, pH 7.9) dried and pressed between filter papers to isolate the integument and to remove the hemolymph and the rest of the tissues. Immediately after pressing the integuments were frozen on dry ice. After washing with ice-cold medium A the tissue was homogenized in 20 ml of medium A by 3 strokes of 20 sec with an Ultraturrax. After stirring during 60 min the homogenate was centrifuged at 48,000 *g* in a Sorvall RC 2B centrifuge. The supernatant was made 3.5 M with respect to ammonium sulfate by adding stepwise crystalline $(NH_4)_2SO_4$ during 60 min. After stirring for 3 hr more the solution was centrifuged at

48,000 *g* and the pellet suspended in 5 ml of medium B (which is medium A without $(NH_4)_2SO_4$). This solution was then stirred gently for 60 min and centrifuged for 15 min at 30,000 *g*. The supernatant was submitted to chromatography on Sephadex G-25 coarse and the desalted protein peak was applied onto DEAE-cellulose, the column was washed with medium B and eluted by a linear gradient of 0.1–0.5 M NH_4Cl. Both the Sephadex and DEAE chromatography were done on identical columns in parallel runs. The NH_4Cl gradient was supplied from one mixer, divided into two parts by a Y-formed distributor and pumped through two identical tubes onto two columns packed with DEAE-cellulose under identical pressure. Titration of the concentration of chloride in the eluate gave the same results in parallel fractions, when these precautions were considered.

2.2. *Preparation of DNA/RNA hybrids*

Preparation of radioactively labelled hybrids was performed as described in detail elsewhere [6]. Calf thymus DNA (Calbiochem, Los Angles) was denatured at 100° for 5 min. After chilling in an ice-bath transcription of this DNA by RNA-polymerase B (prepared from rat liver as described by Seifart et al. [7]) was performed as described in [8]. Under these conditions almost all the synthesized RNA is in form of DNA/RNA hybrids [6]. The hybrids were then treated with RNase and purified from proteins and nucleotides on Sephadex G-75. The resultant solution of hybrids in 0.065 M Tris-buffer, pH 7.9 was made 3 mmolar in Mn^{2+} and used in the assay for hybridase activity.

2.3. *Test for RNase H activity*

Aliquots of 100 μl from the fractions eluted from DEAE-cellulose were incubated with 50 μl of the solution containing hybrids prepared as described above at 37° for 60 min. The reaction was stopped by pipetting aliquots of 120 μl on filter papers, which were immediately immersed in ice-cold 5% trichloroacetic acid (TCA). The filters were then washed 3 times in TCA, 3 times in ethanol (96%), 2 times in ethanol/ether (1:1) and ether, dried and counted as described earlier [9].

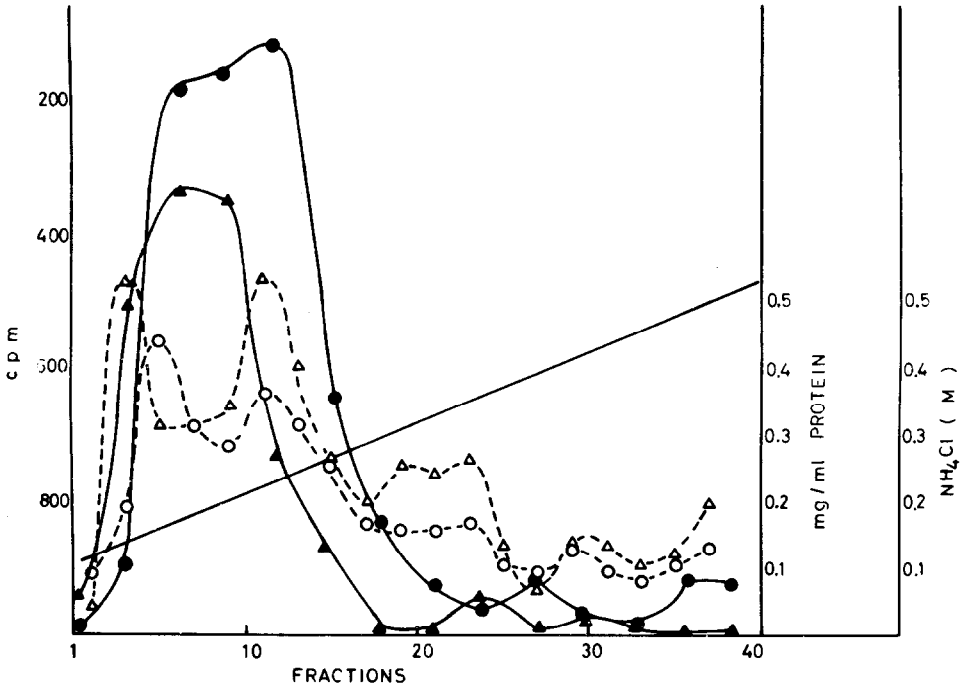

Fig. 1. Hybridase activity of the integument of 7 day old larvae (▲—▲—▲) and white prepupae (●—●—●). Cpm in the ordinate represent radioactivity in acid precipitable material remaining after incubation of labelled DNA/RNA hybrids with the different fractions from DEAE-cellulose (see Methods). Proteins, mg/ml (7 day old larvae △---△---△, white prepupae ○---○---○); NH_4Cl gradient (Molarity Cl^-, ——).

3. Results

The results of the experiments are shown in figs. 1 and 2. In fig. 1 we have compared the activities of hybridase of the integument of 6–7 day old larvae and white prepupae and in fig. 2 those of ligated control animals and of ligated animals injected with β-ecdysone. From figs. 1 and 2 it is evident that hybridase activity is eluted in the region of 0.12 to 0.25 M NH_4Cl showing two maxima of activity at 0.16 M and at 0.23 M NH_4Cl. There is an increase in the activity of hybridase especially of the second peak eluting at 0.23 M in white prepupae as well as in the ligated animals injected with β-ecdysone over the 6–7

Table 1

Substrate	Fraction number 6	12
^{3}H-RNA	95.0%	92.6%
^{3}H-Hybrid	8.2	2.5
^{3}H-Hybrid + AF 013 (200 μg/ml)	95.0	94.4

Comparison of degradation of isolated DNA/RNA hybrids and rat liver nuclear RNA by fractions no. 6 and 12 from the DEAE-cellulose column presented in fig. 1 and effects of AF 013. Radioactivity remaining acid-precipitable after incubation for 60 min at 37° is expressed as percent of control values (incubated with buffer instead of chromatography fractions).

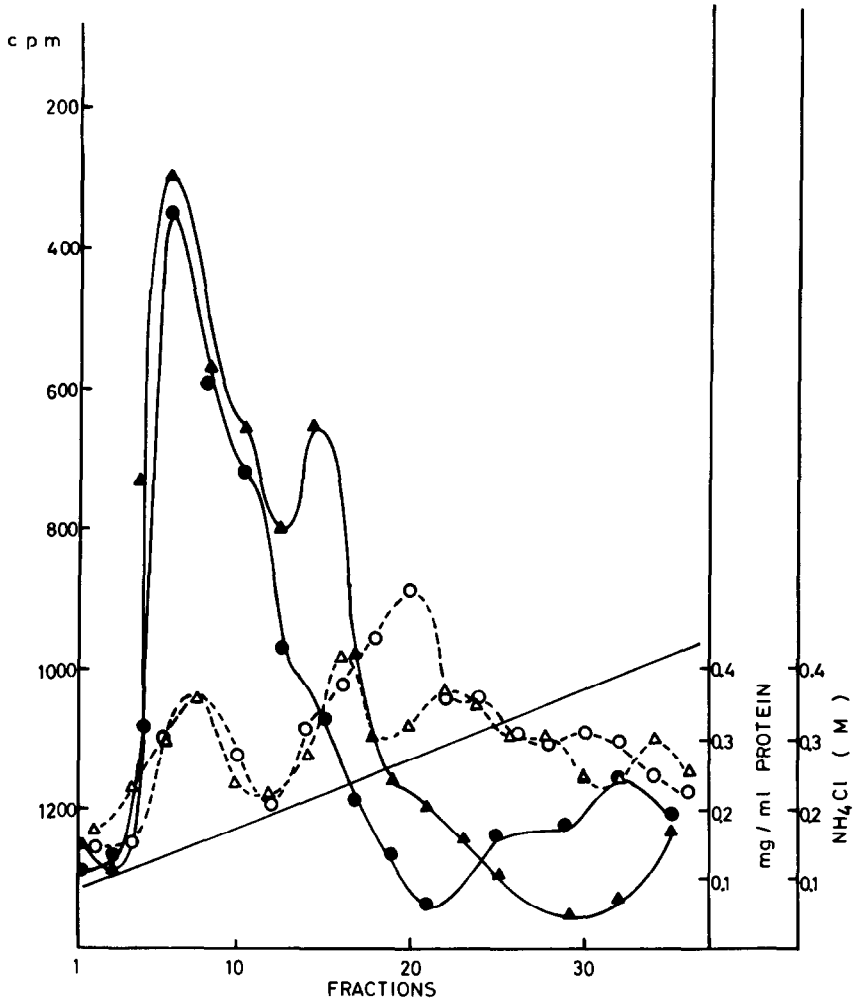

Fig. 2. Hybridase activity of the integument of ecdysone injected (▲—▲—▲) and control animals (●—●—●). See Methods and fig. 1. Proteins, mg/ml (ecdysone-treated animals △---△---△, control animals ○---○---○); NH_4Cl gradient (Molarity Cl^-, ——).

day old larvae or ligated control animals, respectively. These changes in the activity of the hybridase do not reflect changes of possible ribonuclease contaminants of the fractions. This has been excluded by measuring the ribonuclease titer of all the fractions eluted from DEAE-cellulose with radioactive rat liver nuclear RNA as a substrate (see table 1). That we are measuring specific hybridase action is also demonstrated by the fact that the nuclease activity is inhibited by the rifamycin derivative AF 013, which has been shown to inhibit the hybridase activity [10, 6].

4. Discussion

The results presented above clearly show an increase in hybridase activity in white prepupae and in the ligated, ecdysone-depleted animals injected with β-ecdysone. As well documented, ecdysone, either secreted during normal development or injected into hormone-depleted animals rapidly and significantly leads to a many-fold stimulation of RNA synthesis, correlated to the appearance of the biological effects of the hormone i.e. puparium formation (for a review see [10]). Among the species of RNA stimulated by the hormone is DNA-like RNA, which is very probably synthesized *in vivo* by RNA polymerase B. As well known polymerase B *in vitro* preferentially transcribes single stranded DNA leading to the formation of DNA–RNA hybrids which may also be formed *in vivo*. It was therefore of interest to observe that parallel to the increased RNA synthesis an increase in the enzyme degrading DNA/RNA hybrids was seen. It is still premature to causally relate increased transcription with increased hybridase action although such a conclusion is favoured by results obtained from experiments on the effects of hybridase on transcription *in vitro* [12]. Another possibility could be that fluctuations of hybridase activity are correlated to the mechanisms of gene amplification involving reverse transcriptase activity [13, 14]. The inhibitory action of AF 013 on the hybridase suggests some common features of the enzyme with other enzymes inhibited by that drug, such as the reverse transcriptase and the DNA-dependent RNA polymerase of eukaryotes.

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